

Supplemental Text

Methods

This open label phase 2 single arm study examined the activity of prexasertib monotherapy in recurrent high-grade serous or high-grade endometrioid ovarian carcinoma patients without deleterious germline *BRCA* mutation. A documented deleterious germline *BRCA* mutation was obtained in a CLIA-certified laboratory, including but not limited to Myriad Genetics, either by multi-gene panels or individual testing, prior to study enrollment. Variants of uncertain significance (VUS) of *BRCA* were not considered deleterious. Patients with VUS or deleterious germline mutation in other DNA repair genes were eligible. All patients must have had measurable disease, based on RECIST Version 1.1. Biomarker-only disease was not eligible. Other inclusion criteria included an Eastern Cooperative Oncology Group (ECOG) performance status 0–2, and adequate organ and marrow function, defined as haemoglobin ≥ 100 g/L, in the absence of packed red blood cell transfusion within 24 hours prior to study treatment, absolute neutrophil count $\geq 1.5 \times 10^9$ per L, platelet count $\geq 100 \times 10^9$ per L, total bilirubin $\leq 1.5 \times$ the upper limit of normal (ULN), ALT and AST $\leq 3 \times$ ULN, and serum creatinine $\leq 1.5 \times$ ULN or measured creatinine clearance ≥ 45 mL/min per 1.73 m². Patients of reproductive potential must have had serum pregnancy test upon study entry and agreed to use both a barrier method and a second method of birth control during the course of the study and for 4 months after the last dose of prexasertib. Study exclusion criteria included concurrent anticancer therapy, any investigational anticancer therapy or live attenuated vaccine ≤ 4 weeks before first doses of prexasertib (6 weeks for mitomycin C); prior prexasertib or other cell cycle checkpoint kinase inhibitors; central nervous system metastases within 1 year of enrollment; concomitant or prior invasive malignancy within 2 years of enrollment; persistent adverse events from prior anticancer therapy \geq grade 2 per CTCAE Version 4.0; prior history of drug-induced serotonin syndrome or a family history of long-QT syndrome; QTcF interval > 470 msec on screening electrocardiogram; a serious cardiac condition, such as congestive heart failure, New York Heart Association Class III or IV heart disease, unstable angina pectoris, myocardial infarction ≤ 3 months prior to enrollment; arrhythmias that are symptomatic or refractory to medical intervention ≤ 28 days prior to enrollment. Additional exclusion criteria included history of human immunodeficiency virus on combination antiretroviral therapy. The recommended phase 2 dose of 105 mg/m² prexasertib was administered intravenously over 1 hour once every 14 days. Administration of prexasertib may have been delayed up to 7 days due to holidays, inclement weather, conflicts, toxicity or similar reasons. For safety reasons, prexasertib was not administered in less than a 14-day interval.

Study Oversight

The study has been conducted in accordance with ethical principles that have their origin in the Declaration of Helsinki and are consistent with the International Council on Harmonization guidelines on Good Clinical Practice, all applicable laws and regulatory requirements, and all conditions required by a regulatory authority and/or Institutional Review Board (IRB). The study protocol was reviewed and approved by the IRB of the Center for Cancer Research, National Cancer Institute, USA. Written informed consent was obtained from all patients.

Correlative studies

Pretreatment tumour biopsies and paired blood samples were collected at baseline and on cycle 1 day 15 (6–24 hours after the end of prexasertib infusion) as described.¹ Percutaneous biopsies were obtained by interventional radiologists under CT or ultrasound guidance using local anesthesia. Samples were processed immediately in real time into optimal cutting temperature compound and stored at -80°C , then cut and stained immediately prior to use.² Optimal quality of tissue was defined as core biopsy samples with solid tissue areas containing at least 50% tumour cells and less than 25% necrosis.² Tissue area was measured and prepared.³

Homologous recombination deficiency (HRD) definition

HRD analysis was performed using pretreatment fresh frozen core biopsy. HRD was defined as *RAD51C* or *BRCA1* promoter hypermethylation or a deleterious germline or somatic mutation identified by BROCA-HR sequencing present in $>10\%$ of the neoplastic fraction in one of the following genes: *ATM*, *ATR*, *BARD1*, *BLM*, *BRCA1*, *BRCA2*, *BRIP1*, *CDK12*, *CHEK2*, *MRE11A*, *NBN*, *PALB2*, *RAD51C*, *RAD51D*, *SLX4*, *RBBP8*, *XRCC2*.⁴

Copy number variation (CNV) analysis

Total genomic DNA (gDNA) was isolated from a baseline core biopsy, using the Qiaamp DNA microkit (Qiagen, Germantown, MD, USA). DNA concentration and quality was estimated by NanodropTM and 10 ng was used as template for each reaction in a multiplex Q-PCR format.^{5,6} gDNA from the epithelial ovarian carcinoma cell line A2780, known to have normal two copies of *CCNE1* and *CCND1*, was used as normal control. CNV analysis for *CCNE1* was performed using three different Taqman[®] MGB probes (ThermoFisher scientific, Waltham, MA, USA) targeting an internal region of exon7 (Hs02527940), intron8-exon9 junction (Hs01268776), and intron 4

(Hs07137484), respectively.^{5,7,8} Copy numbers > 3 was considered as *CCNE1* gene amplification and copy numbers between 2·1 and 3 was considered as copy number gain. CNV analysis for *CCND1* was performed with MGB probes targeting the junction of intron4-exon5 (Hs02353610) or intron 3 (Hs03803699) or exon3 (Hs01133305).⁹ A VIC labelled TAMRA™ probe for RNaseP (Thermofisher scientific) was used as reference control for both probe sets.⁵ Ct values were then used to estimate copy numbers using the Copycaller™ software (Applied Biosystems, Carlsbad, CA, USA) in each patient, using A2780 as a normal control. Copy numbers > 3 was considered as *CCND1* gene amplification.

RNA isolation and sequencing

Total RNA was isolated from a pretreatment core biopsy, using the RNeasy microkit (Qiagen). Quality of RNA was evaluated using the Agilent Bioanalyzer 2100 and RNA integrity number (RIN) values were ensured to be > 8·0. For total RNA sequencing (RNA-Seq), each sample (20-100 ng) was preprocessed with NEBnext rDNA depletion kit (New England Biolabs, Ipswich, MA, USA) to remove ribosomal RNA, barcoded and pooled to ensure at least 100 million reads per sample on a HiSeq3000 sequencing system (Illumina, San Diego, CA, USA). Reads were aligned against the Human reference genome Hg38 and gene expression data was generated as counts per million mapped reads (CPM) values. Quality check of sample and sequencing outputs were performed by the Center for Cancer Research (CCR) sequencing facility and CCR collaborative bioinformatics resource at National Cancer Institute, Bethesda, MD, USA and ensured to be within recommended parameters. RNA-Seq data on normal ovary tissues were obtained from the Genotype-Tissue Expression project (GTEx project, NIH; <https://www.gtexportal.org/home/>, last accessed on 8/29/2017)¹⁰ as Reads Per Kilobase of transcript per Million mapped reads (RPKM) values then converted to CPM values using the formula $RPKM = CPM / L$, where L is the exonic length in kilobases. Log2 of CPM values were used throughout.

Immunohistochemistry analysis

Hematoxylin and eosin stained slides were evaluated to confirm diagnosis. Protein expression of cyclin E1 was examined by immunohistochemistry using available unstained slides with 5 µm fresh frozen biopsy sections.^{11,12} Rabbit polyclonal antibodies were purchased from Abcam (Cambridge, MA, USA) and used per manufacturer's recommendation. Antibody treatment and staining were performed using the Vectastain ABC kit and Impact™ DAB substrate (Vector laboratories, Burlingame, CA, USA) per manufacturer's instruction. Slides were examined at X15 and X250 by an experienced pathologist (MM) who was not informed of the results obtained from mRNA measurements. Staining was positive when localized to the nucleus. Cyclin E staining was defined as presence of "negative", "positive" or "strongly positive", depending on the intensity and percentage of the cells showing a nuclear staining pattern.^{11,12}

Circulating tumor cells (CTC) enumeration and characterization

Peripheral blood samples were drawn into EDTA tubes before the first dose of treatment and cycle 1 day 15 (6-24 hours after the end of prexasertib infusion). Cells were stained with PE-conjugated anti-human epithelial cell adhesion molecule (EpCAM) Ab (clone HEA-125, Miltenyi Biotec, San Diego, CA, USA) then EpCAM-positive cells were enriched using anti-PE magnetic beads (Miltenyi Biotec) and quantified by multiparameter flow cytometry.¹³⁻¹⁵ Viability was defined by the absence of aqua fluorescent reactive dye (Life Technologies, Washington, DC, USA) staining, and analysis was restricted to nucleated cells by gating on Hoechst 33342 (Life Technologies)-positive cells. CTCs were identified as viable, nucleated, EpCAM-positive cells that did not express the common leukocyte antigen CD45 (APC-Cy7-CD45 clone HI30, BioLegend, San Diego, CA, USA).¹³⁻¹⁵ Patients were divided into CTC negative (< 2 CTCs) or positive (≥ 2 CTCs) groups.^{16,17} Comparisons of parameters at baseline or the change between cycle 1 day 15 and baseline with respect to response or PFS exceeding 6 months were performed using a Wilcoxon rank sum test. The significance of the change in CTC from baseline to cycle 1 day 15 was determined using a Wilcoxon signed rank test.

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FIGURE LEGENDS

Supplementary Figure S1. Progression free survival

Median potential follow-up of 24 evaluable patients was 16·7 months (IQR: 13-26·3 months). Median PFS was 7·4 months (95% CI: 2·1-9·4 months) with 54·2% of 6 month PFS (95% CI: 32·7-71·4%) and 20·8% of 12 month PFS (95% CI: 7·6-38·5%). For PFS events, 19 had a progression event and one had death on study due to tumour progression.

Supplementary Figure S2. CCNE1 and CCND1 copy number variations (CNV)

Results of 24 patients with baseline (A) CCNE1 and (B) CCND1 copy number analysis are shown. CNV analysis for CCNE1 was performed and graphed for each patient. The dotted line indicates the normal copy number of 2. The red dot indicates patients with PR by RECIST v1·1 and red arrows indicate those receiving drug at data lock. Blue: platinum-sensitive; grey, resistant; yellow: refractory disease. Study ID 48's core biopsy sample consisted of normal liver tissue without optimal quality of tumor tissues.

Supplementary Figure S3. Relationship between CCNE1 copy number and mRNA expression

Each individual patient is marked as x (n=23). CCNE1 mRNA levels tend to increase with copy number gain or amplification; all cases with either CCNE1 amplification (five) or copy number gain (seven) had CCNE1 mRNA upregulation. Study ID 48's data was not included to this figure, which yielded normal CCNE1 copy number and -0·1 log2 CPM value.

Supplementary Figure S4. Circulating tumor cells (CTCs)

23 patients with baseline CTCs are shown separately for EpCAM-positive CTCs based on (A) the RECIST response (PR vs. SD/PD; p=0·28) and (B) PFS (< 6 months vs. ≥ 6 months; p=0·42). Patients were divided into CTC negative (< 2 CTCs, black open circle) or positive (≥ 2 CTCs, black dot) groups. The dotted line indicates the median number of CTCs. At least one CTC was isolated from the peripheral blood of over 90% (21/23) of the patients (range 0-20 CTCs).

(C) 22 patients with paired CTCs (pretreatment and cycle 1 day 15) are shown. There was an increase in CTC of 4·5 over baseline (p=0·016 by Wilcoxon signed rank test).

Abbreviations: PR = partial response, SD = stable disease, PD = progression of disease

Supplementary Table S1. Hereditary breast ovarian cancer syndrome family history criteria (modified from NCCN Clinical Practice Guidelines 2013)¹⁸

Personal history of breast cancer and one or more of the following:	Personal history of ovarian cancer and one or more of the following:
<ol style="list-style-type: none"> 1. A family member with a known deleterious <i>BRCA1</i> or <i>BRCA2</i> mutation. 2. Diagnosed at age ≤ 50 year-old with ≥ 1 first, second, or third degree relative with invasive breast cancer and/or DCIS diagnosed at any age. 3. Diagnosed at any age with ≥ 1 first, second, or third degree relative with invasive breast cancer and/or DCIS, diagnosed at age ≤ 50 year-old. 4. Diagnosed at any age with ≥ 2 first, second, or third degree relative with invasive breast cancer and/or DCIS diagnosed at any age. 5. Diagnosed at any age with ≥ 1 first, second, or third degree relative with epithelial ovarian cancer diagnosed at any age. 6. Diagnosed at any age with ≥ 2 first, second, or third degree relative with pancreatic cancer and/or aggressive prostate cancer (Gleason score ≥ 7) diagnosed at any age. 7. ≥ 1 first, second, or third degree male relative with invasive breast cancer and/or DCIS diagnosed at any age. 8. Individual ethnicity associated with higher mutation frequency (i.e. Ashkenazi Jewish). 	<ol style="list-style-type: none"> 1. A family member with a known deleterious <i>BRCA1</i> or <i>BRCA2</i> mutation. 2. ≥ 1 first, second, or third degree relative with invasive breast cancer and/or DCIS diagnosed at any age. 3. ≥ 2 first, second, or third degree relative with pancreatic cancer and/or aggressive prostate cancer (Gleason score ≥ 7) diagnosed at any age. 4. ≥ 1 first, second, or third degree male relative with invasive breast cancer and/or DCIS diagnosed at any age. 5. Individual ethnicity associated with higher mutation frequency (i.e. Ashkenazi Jewish).

Abbreviations: DCIS: ductal carcinoma in situ

Supplementary Table S2. BROCA-HR analysis results

Study ID	Tumor type	Platinum-sensitivity	PFS (months) as of 7/1/2017	Best response as of 7/1/2017	Mutation in genes of HRR pathway	BRCA1 Promoter methylation	RAD51C Promoter methylation	HRD positivity	Other mutations
1	HGSOC	Platinum-sensitive	9	PR		No	No	No	
10	HGSOC	Platinum-sensitive	2	PD		No	No	No	POLE c.2760delC (p.T920fs), 0.48 vaf, somatic
17	HGSOC	Platinum-sensitive	13	PR	somatic CDK12 c.2700G>A (p.W900X), 0.79 vaf	No	No	Yes	
35	HGSOC	Platinum-sensitive	8.5	SD		No	No	No	
48	HGSOC	Platinum-sensitive	2	PD		N/A	N/A	N/A	
4	HGSOC	Platinum-resistant	9	PR		N/A	N/A	N/A	
5	HGSOC	Platinum-resistant	2	PD		No	No	No	PIK3CA c.3140A>G (p.H1047R), 0.75 vaf, somatic
6	HGSOC	Platinum-resistant	8	SD		No	No	No	
7	HGSOC	Platinum-resistant	2	PD		N/A	N/A	N/A	
14	Mixed HGSOC and clear cell*	Platinum-resistant	2	PD	suspected for germline BRCA2 c.7878G>C (p.W2626C), 0.65 vaf	No	No	Yes	
23	HGSOC	Platinum-resistant	4	SD		No	No	No	
24	HGSOC	Platinum-resistant	4	SD	somatic SLX4 c.5229dupG (p.Q1744fs), 0.3 vaf	No	No	Yes	
28	HGSOC	Platinum-resistant	10	SD		N/A	N/A	N/A	
29	HGSOC	Platinum-resistant	16.5+	PR		N/A	N/A	N/A	
31	HGSOC	Platinum-resistant	7.5	PR		No	No	No	
33	HGSOC**	Platinum-resistant	16+	SD		Yes	No	Yes	
36	HGSOC	Platinum-resistant	2	PD		No	No	No	
37	HGSOC	Platinum-resistant	13.5+	PR		No	No	No	
38	HGSOC	Platinum-resistant	3	SD		Yes	No	Yes	
40	HGSOC	Platinum-resistant	12.5+	PR		No	No	No	
41	HGSOC†	Platinum-resistant	3.5	uPR		No	No	No	
46	HGSOC	Platinum-resistant	2	PD		Yes	No	Yes	
47	HGSOC	Platinum-resistant	7.5	SD		No	No	No	
42	HGSOC	Platinum-refractory	9.5	SD		No	No	No	

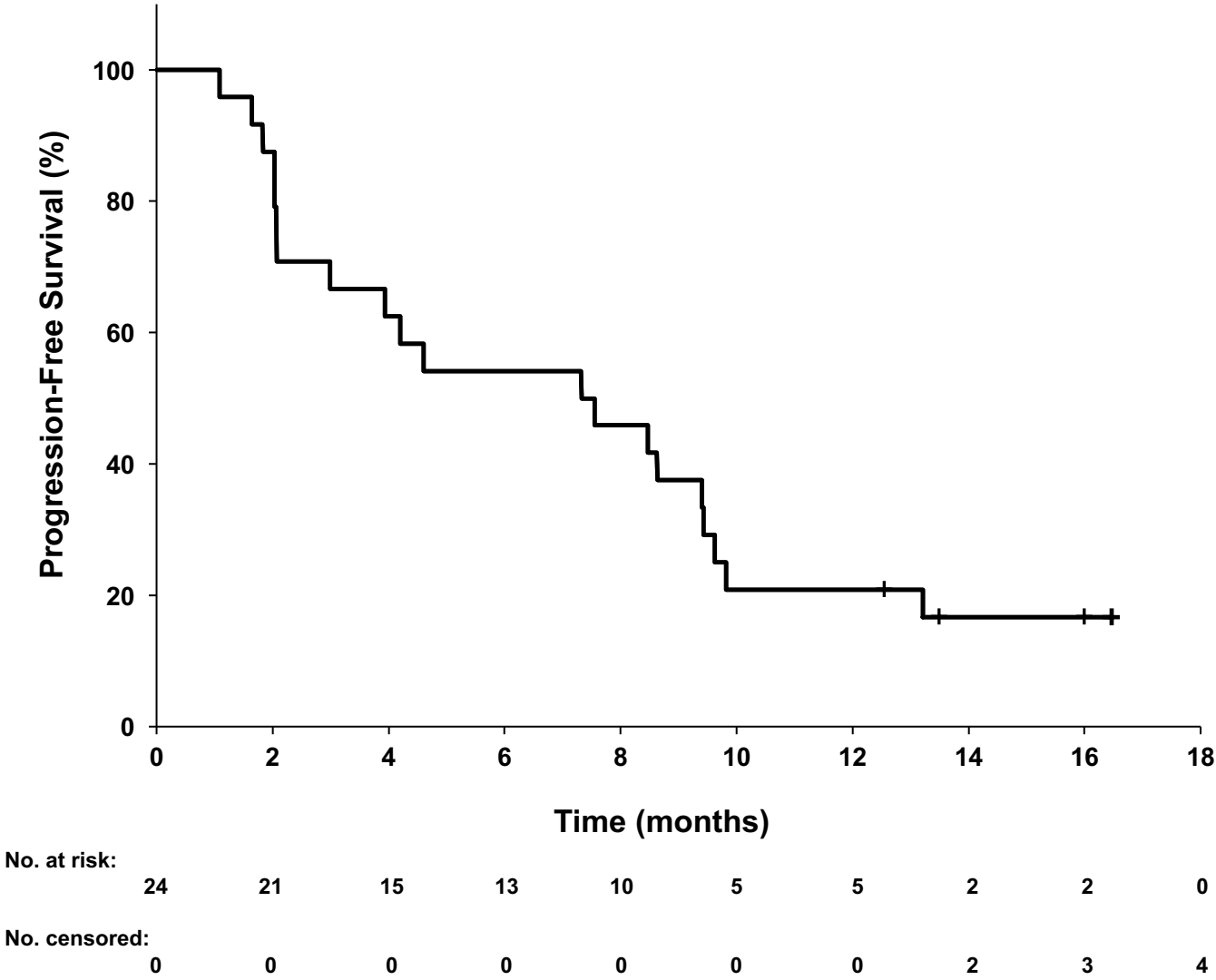
* A former commercial BRCA testing in 2013 also showed suspected deleterious *BRCA2* mutation and the patient was eligible for study entry criteria in 2015. This patient received olaparib for nine months prior to enrollment.

** All but this patient had tested germline *BRCA* mutation prior to study enrollment, and testing by BROCA-HR revealed no germline and somatic *BRCA* mutations.

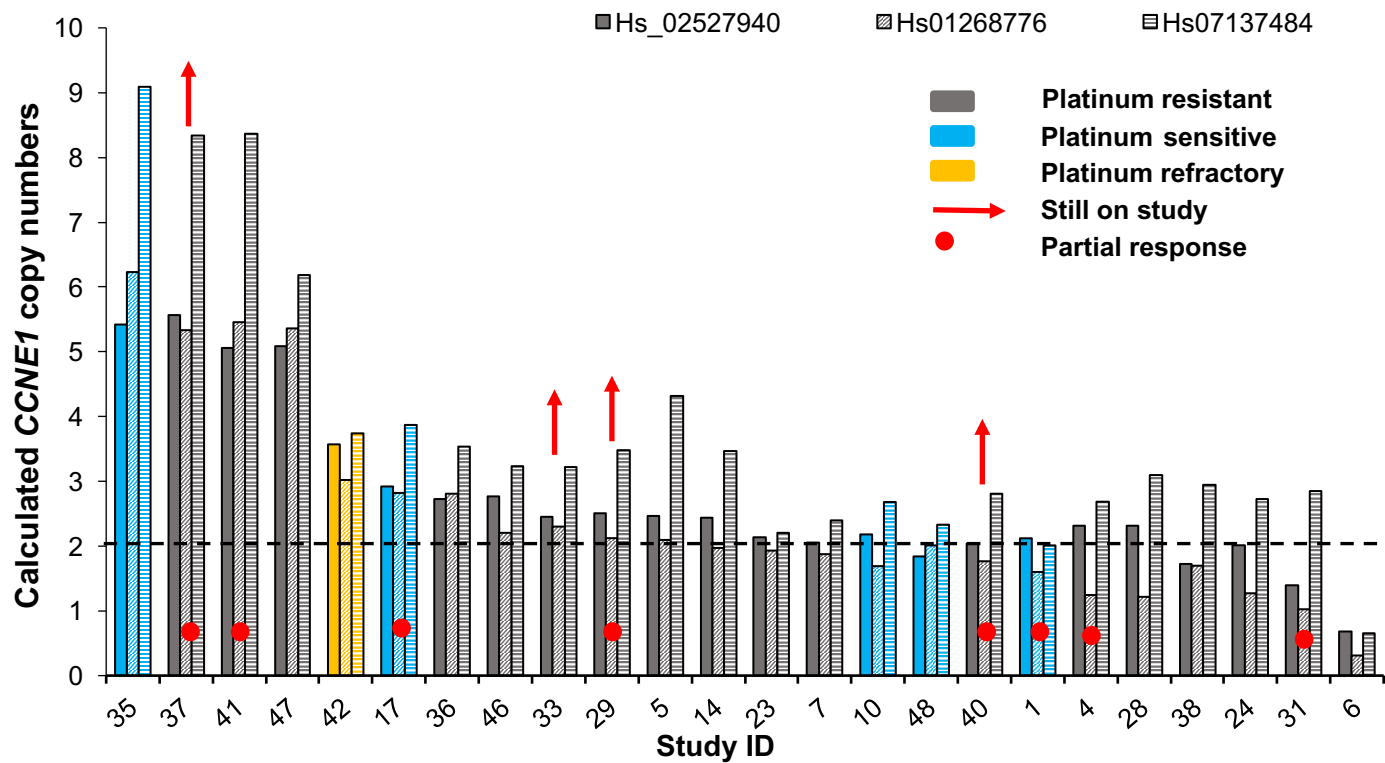
† This patient achieved PR but she withdrew during cycle 4 due to grade 2 fatigue and travel inconvenience, thus PR was not confirmed.

Study ID 48's core biopsy sample consisted of normal liver tissue with suboptimal quality of tumor tissues.

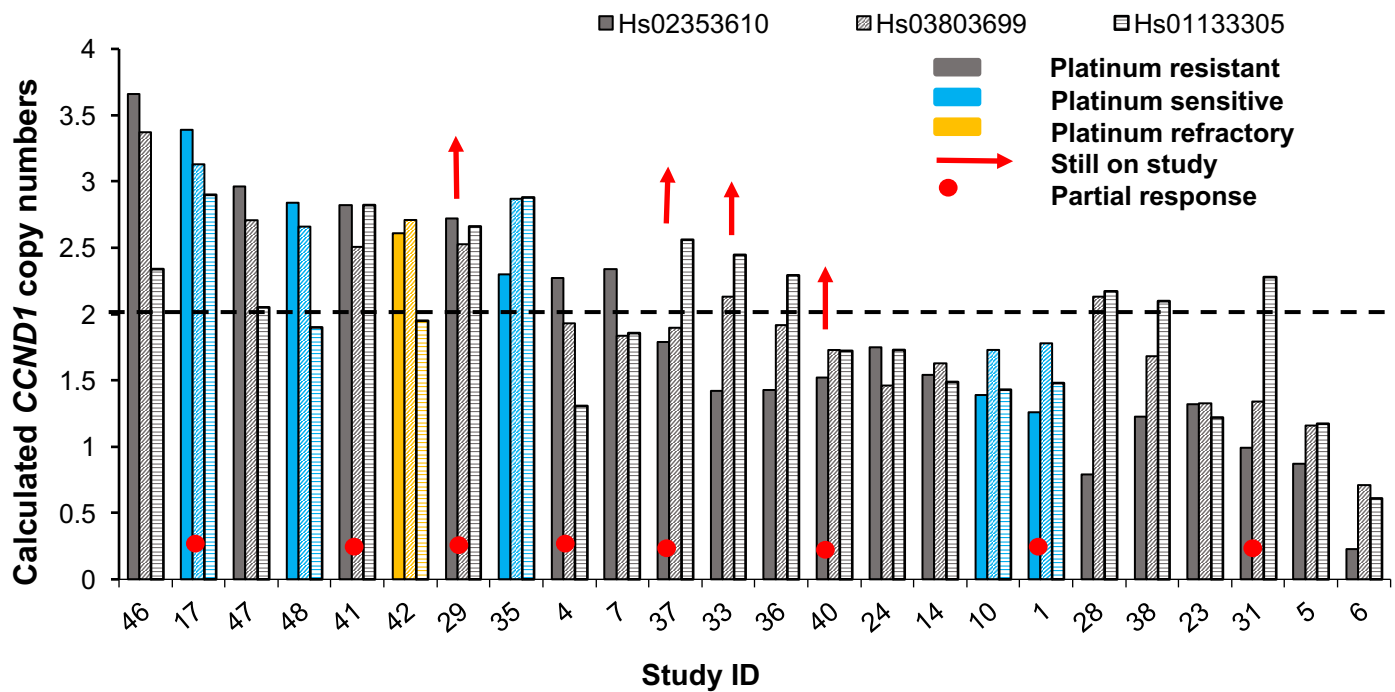
Abbreviations: PFS = progression free survival, HGSOC = high grade serous ovarian cancer, HRR = homologous recombination repair, HRD = homologous recombination deficiency, PR = partial response, uPR = unconfirmed PR, SD = stable disease, PD = progression of disease, N/A = not applicable due to suboptimal quality of tumor samples.

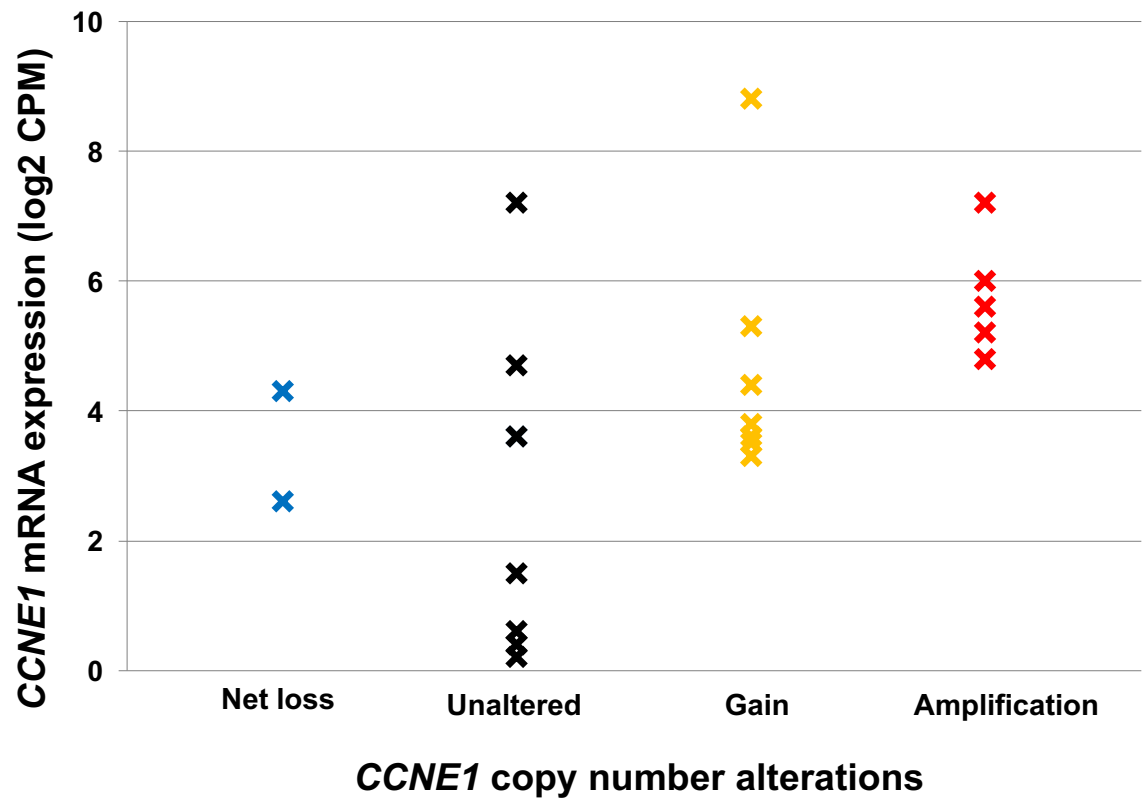


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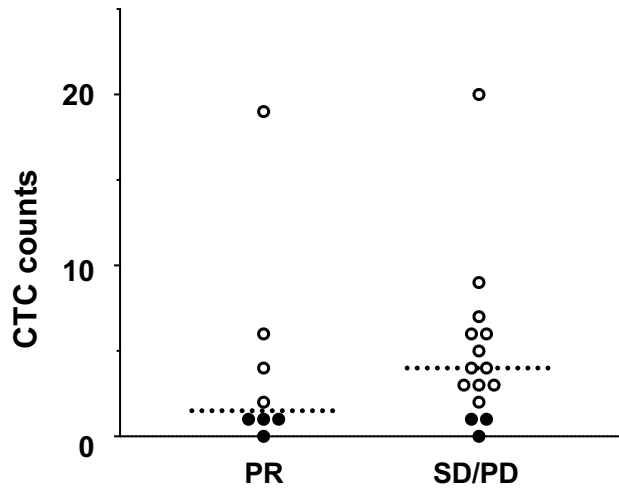


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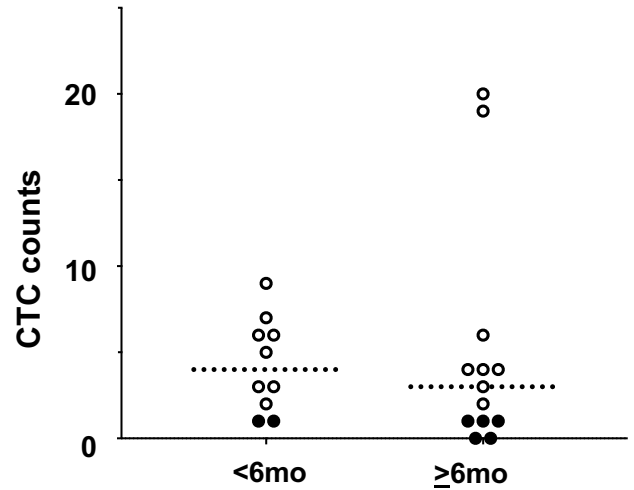




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